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IN VITRO BONE FORMATION ASSOCIATED WITH APATITE COATED POLYLACTIDE

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Abstract

Bone formation onto poly(L-lactide), which was plasma-spray coated with various quantities of hydroxyapatite (0%, 15%, 36% and 100% coverage), was investigated in an *in vitro* assay. Rat bone marrow cells were grown on the different coatings and the cellular response and elaborated extracellular matrix was examined at the light and electron microscopical level after 1, 2, 4 and 8 weeks of culture. Proliferation of cells into multilayers was seen on the 0%, 36% and 100%, but not on the 15% coatings. Coinciding with this was the sparse formation of extracellular matrix on the latter, and its abundant appearance on the former three coatings. Scanning and transmission electron microscopy revealed a mineralized extracellular matrix on the 100% and 36% coatings after 2 and 4 weeks, respectively, and on the 15% coating after 8 weeks. Mineralization was not observed on uncoated poly(L-lactide). At the interface between hydroxyapatite and the mineralized extracellular matrix, one or more electron dense layers were frequently observed, which showed morphological similarities with structures between these two entities *in vivo*. The results of this *in vitro* study show that, in the model used, hydroxyapatite is required to obtain the elaboration of mineralized extracellular matrix on poly(L-lactide).

Key Words: Polylactide, hydroxyapatite, coatings, composite, bone formation, *in vitro*, interface, osteoblast, mineralization, scanning electron microscopy, transmission electron microscopy.

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Introduction

An optimal bone replacement material should be bone-bonding, bioactive, mechanically strong, biodegradable and participate, at least partially, in the normal turnover of bone. Currently used biomaterials, however, do not fulfill all these requirements. Existing implant materials used for bone replacement can be divided into calcium phosphates, metals, polymers and composites. From the former group, hydroxyapatite is the most frequently used implant material because of its bone-bonding ability (Hench *et al.*, 1972; Osborn and Newesely, 1980; Jarcho, 1981; van Blitterswijk *et al.*, 1990). When implanted into bone, it can form a physico-chemical bond with this tissue. Furthermore, hydroxyapatite is degradable to a certain extent but, due to its brittleness and low fatigue resistance, it cannot be used as a load bearing implant material. With regard to polymers, polylactide is an implant material that is used in bone pins, screws and fixation plates (Rokkanen *et al.*, 1985, 1991; Bos *et al.*, 1987; Rozema *et al.*, 1988). It has good biocompatible characteristics (Majola *et al.*, 1991; van Sliedregt *et al.*, 1992) and the degradation properties and elasticity modulus can be varied with the molecular weight and the choice of L- and/or D-enantiomer (Kulkarni *et al.*, 1971). A disadvantage of polylactide, however, is that it does not form a bond with bone tissue and is therefore not a "bioactive" material.

The combination of different biomaterials in a composite material has given novel dimensions to the development of new structural implants. Several *in vivo* and *in vitro* experiments have been described in which bone formation onto various calcium phosphate/polymer composites was studied (Nelson *et al.*, 1977; Bonfield *et al.*, 1986; Higashi *et al.*, 1986; Scalzo *et al.*, 1989; Tarrant and Davies, 1989). The potential advantage of composites made of hydroxyapatite and polylactide is that a degradable, bioactive and mechanically strong material is formed. These material characteristics will be influenced by the type of polylactide chosen and the amount of hydroxyapatite present in the composite material. The percentage of hydroxyapatite in the composite can be varied, depending on the required mechanical properties. Other studies (Verheyen *et al.*, 1991) have

indicated that > 50% w/w (weight/weight) hydroxyapatite added to polylactide resulted in materials that were too brittle, whereas < 20% w/w hydroxyapatite added to polylactide was not expected to significantly increase the bone bonding properties. To investigate the influence of composite constitution, and particularly the amount of hydroxyapatite surface available, on its bone-bonding ability, poly(L-lactide) was coated with different covering percentages of hydroxyapatite. In order to mimic a composite material and to improve reproducibility, we chose a plasma sprayed hydroxyapatite coating onto poly(L-lactide).

The aim of this study was to examine the influence of different percentages of hydroxyapatite covering on poly(L-lactide), on bone formation *in vitro*. This was examined in an *in vitro* bone forming system which has previously been used to study interfacial reactions with titanium, hydroxyapatite, and other biomaterials (Davies, 1990; Davies *et al.*, 1991; de Bruijn *et al.*, 1991, 1992a, b). The mineralization process was followed in time at both the light microscopical and ultrastructural level.

Materials and Methods

Hydroxyapatite/poly(L-lactide) composites

Poly-L-lactide (PLA). For the *in vitro* experiments, PLA cylinders with a diameter of 13 mm were machined from a block of high molecular weight (927 kD) as-polymerized PLA (Purac bv, Gorinchem, The Netherlands), and 200 μm thick discs were cut using a diamond saw (Buehler low speed diamond saw, Isomet®).

Hydroxyapatite. Different ratios of hydroxyapatite were applied onto the PLA materials using the plasma spray method (Figs. 1a-c). Using scanning electron micrographs, the amount of hydroxyapatite covering was quantitatively measured with a Vidas Image Analysing System and the percentages of coating were 0%, 14.6 $\pm$ 7.6% (15%), 36.3 $\pm$ 14.2% (36%) and 100%. Figure 1d shows the X-ray diffraction pattern of the hydroxyapatite coatings. The molecular weight of the PLA before and after plasma spraying was measured by Purac bv (Gorinchem, The Netherlands) and was 927 and 570 kD, respectively. The samples were gas sterilized using ethylene oxide prior to the cell culture experiments.

Cell culture

An osteogenic rat bone marrow culture (Maniatopoulos *et al.*, 1988) was used for the *in vitro* experiments as described previously (de Bruijn *et al.*, 1991). Briefly, third passage rat bone marrow cells obtained from femora of young adult male Wistar rats (100-120 g) were cultured on the materials at a concentration of $1x10^4$ cells/cm^2. The culture medium was composed of alpha-minimal essential medium (α-MEM, Gibco) supplemented with 15% foetal calf serum (Gibco), penicillin/streptomycin, and freshly prepared 10 mM ß-glycerophosphate, 50μg/ml ascorbic acid and 10^{-8} M dexamethasone. Cultures were placed in an incubator at 37°C in a humidified atmosphere of 90% air and 10% CO_2 and refed every 48 hours. To examine the influence of culture medium on the coatings, control specimens with a 100% hydroxyapatite coating were incubated in medium without cells. After 1, 2, 4 and 8 weeks, cultures were processed for light, scanning and transmission electron microscopy.

Light microscopy

Specimens were fixed in 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.4, 4°C) for 30 minutes, dehydrated through a graded series of ethanol and embedded in glycol methacrylate. Semi-thin sections were cut and then stained with toluidine blue or alcian blue.

Alkaline phosphatase cytochemistry. Alkaline phosphatase activity was detected using the Azo-dye method of Gomori. The substrate solution was composed of 2 mg/ml α-naphthyl phosphate and 1 mg/ml Fast Blue RR salt, dissolved in 0.1 M Na-barbiturate buffer pH 9.2. Fixed cells were incubated for 10 minutes in the substrate solution and then thoroughly rinsed in tap water. Specificity for alkaline phosphatase activity was determined by incubating cells with a control substrate solution in the absence of α-naphthyl phosphate.

Electron microscopy

Transmission electron microscopy (TEM). Cells were fixed according to the light microscopical procedures and after rinsing in 0.14 M sodium cacodylate buffer, pH 7.4, postfixation was carried out in an aqueous solution of 1.5% potassium ferrocyanide and 1% OsO_4 for 16 hours at 4°C. Specimens were dehydrated through a graded series of ethanol and embedded in Epon. Ultra-thin sections were prepared on a LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips EM 201 or 400 operated at 80 kV.

Scanning electron microscopy (SEM). Specimens were fixed and dehydrated according to the routine TEM procedure and air dried with tetramethylsilane (Merck). A layer of gold was sputter coated onto the specimens with a Balzers sputter coater model MED 010 and they were examined in a Philips S 525 SEM operated at an accelerating voltage of 15 kV.

Results

Bone marrow culture

Cells showed a high alkaline phosphatase activity on all materials, independent of the amount of hydroxyapatite covering. However, a difference in cell proliferation was seen on the different coatings. Figure 2 shows a scanning electron micrograph of the different hydroxyapatite coverings after 1 week of cell culture. The individual particles of the hydroxyapatite coating were still visible on the 15% coated PLA, while the other materials were fully covered by a cell multilayer. In this multilayer, abundant fibrillar material was observed, while this was sparse on 15% coated PLA. The

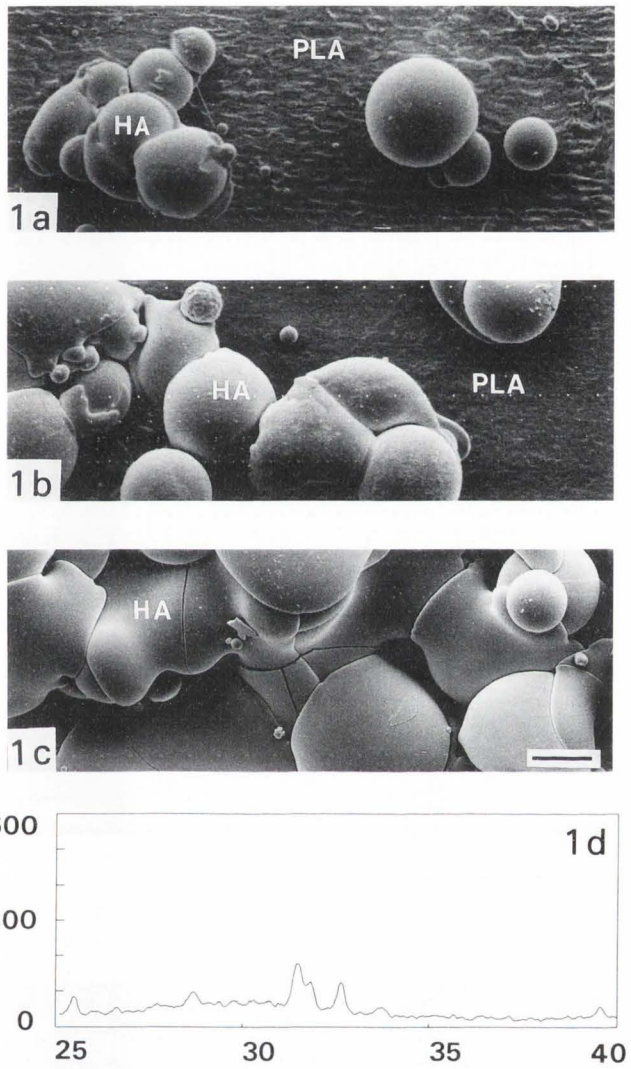


Figure 1. Poly(L-lactide) (PLA) discs with an increasing amount of hydroxyapatite (HA) covering: (**a**) 15%, (**b**) 36%, (**c**) 100%. The powder X-ray diffractogram of the hydroxyapatite coating, showing low peaks and peak broadening, is indicative of an amorphous phase (**d**). Bar = 12.5 μm.

hydroxyapatite surface of all samples showed early signs of degradation after only 1 week of culture, indicated by the presence of small cavities and a porous appearance (Fig. 3).

To examine the time period in which mineralized extracellular matrix was formed on the different coatings, specimens were examined with LM, SEM and TEM. When stained with either toluidine blue or alcian blue, a basophilic line was observed on the hydroxyapatite coated parts of the specimens and not on the PLA. It was present after 1 week on both 36% and 100% coated PLA, but only after 8 weeks on 15% coated PLA. SEM and TEM showed a mineralized extracellular matrix from 2 and 4 weeks onwards on the 100% and 36%

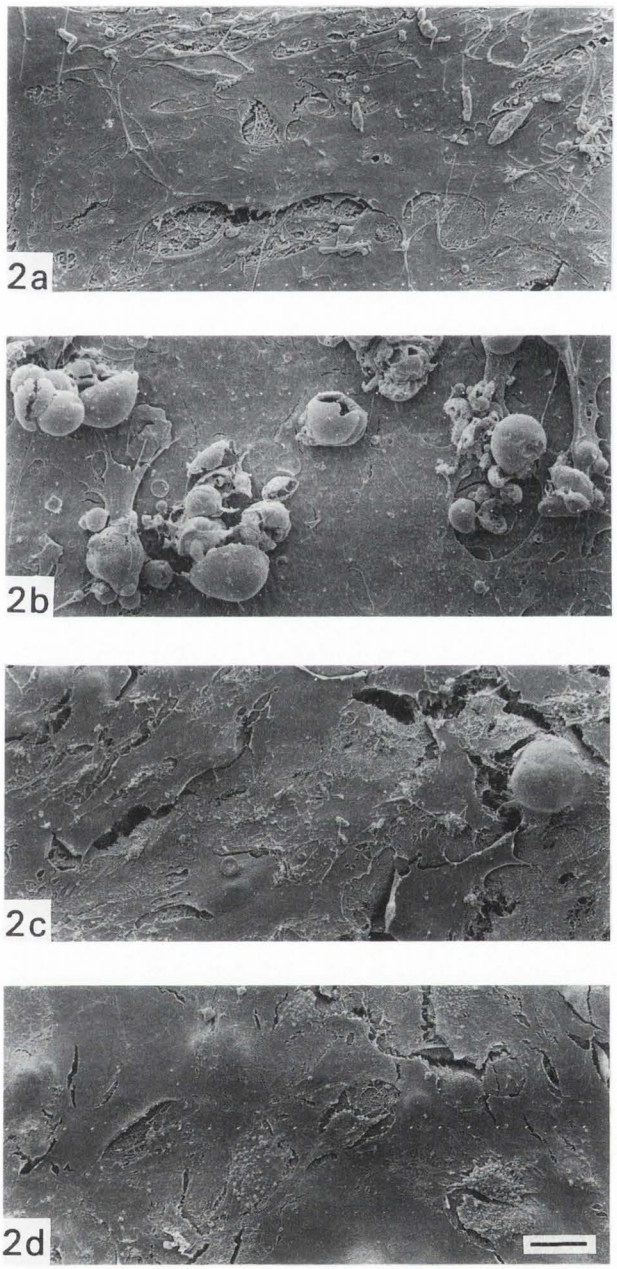


Figure 2. Cells cultured for 1 week onto the various hydroxyapatite coatings as seen in Figure 1. (**a**) uncoated, (**b**) 15% coated PLA, (**c**) 36% coated PLA and (**d**) 100% coated PLA. A multilayer and abundant extracellular matrix is present on 0%, 36% and 100% coated PLA. Note the plasma-sprayed hydroxyapatite particles on 15% coated PLA, that are not fully overgrown with cells. Bar = 10 μm (a), 23 μm (b, c, d).

coated PLA respectively (Fig. 4), and at 8 weeks on the 15% coated PLA. An intimate contact was observed between the mineralized collagen fibres and the hydroxyapatite surface (Fig. 5). After 8 weeks of culture, 100% coated PLA was fully covered with globular, mineralized

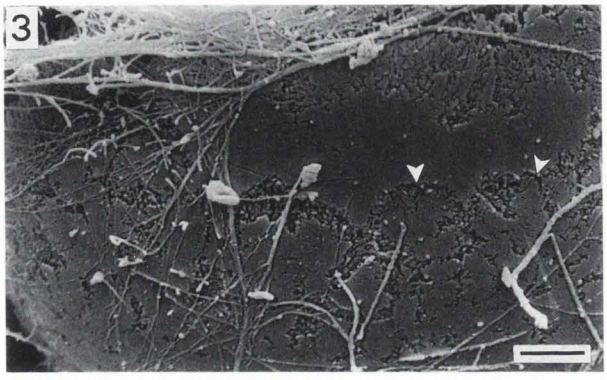
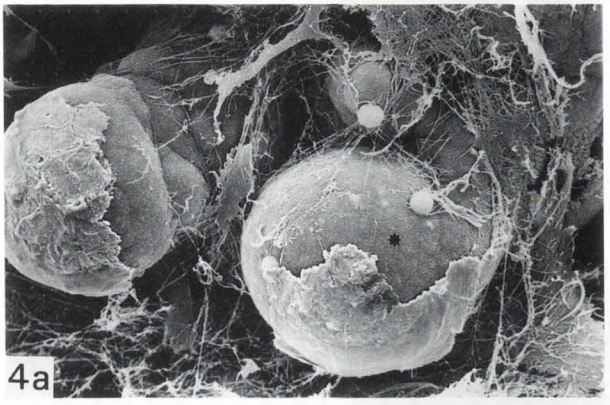


Figure 3. Degrading hydroxyapatite particle from 15% coated PLA, onto which fibrillar material is deposited. Note small cavities (arrowheads) in the hydroxyapatite particle, suggestive for degradation. 1 week culture, Bar = 1.8 μm.



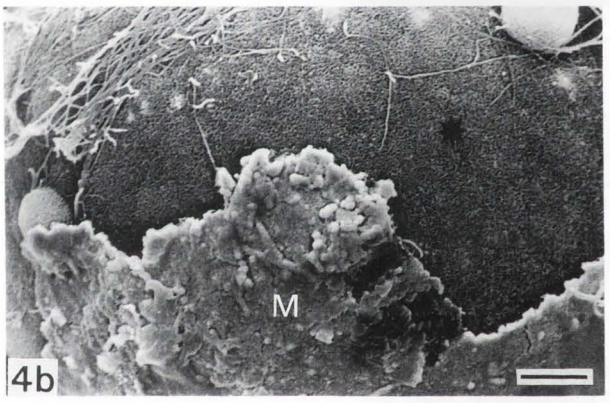
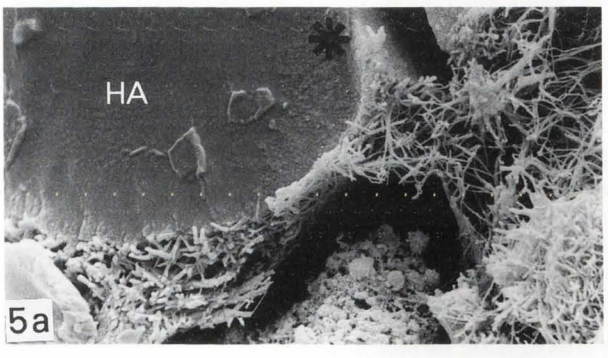


Figure 4. 100% coated PLA after 2 weeks cell culture. Note the globular mineralized material (M) that is formed onto the plate-like crystals (asterisk), covering the hydroxyapatite surface. Bar = 5.9 μm (a), 1.9 μm (b).



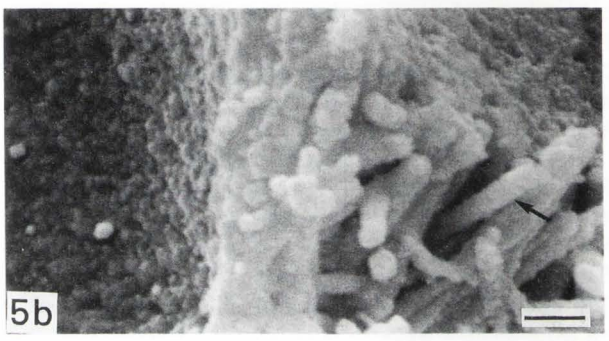


Figure 5. (**a**) Scanning electron micrograph of 100% coated PLA onto which a mineralized extracellular matrix is deposited. Note a gradual transition (asterisk) from the hydroxyapatite (HA) particle towards the mineralized extracellular matrix. (**b**) Higher magnification of (a), showing mineralized collagen fibres (arrow). Bar = 3.0 μm (a), 0.4 μm (b).

Figure 6. Globular, mineralized accretions (arrows) formed on the 100% hydroxyapatite coating in which collagen fibres are attached. Eight weeks of culture. Bar = 2.94 μm.

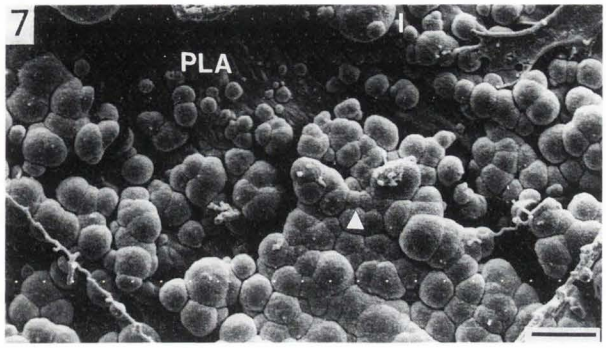
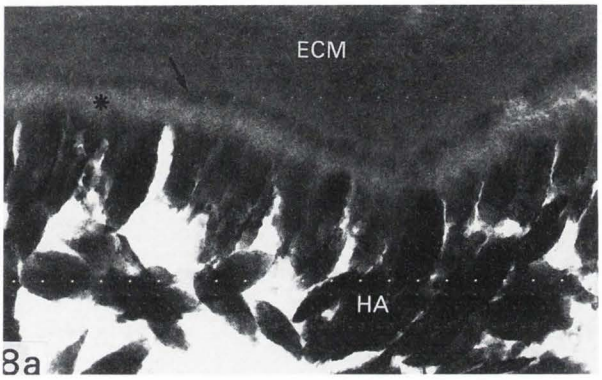


Figure 7 (above). Deposition of mineralization globules (▲) onto the PLA surface of a 36% hydroxyapatite coated sample, after 1 week of culture. Bar = 6.3 μm.



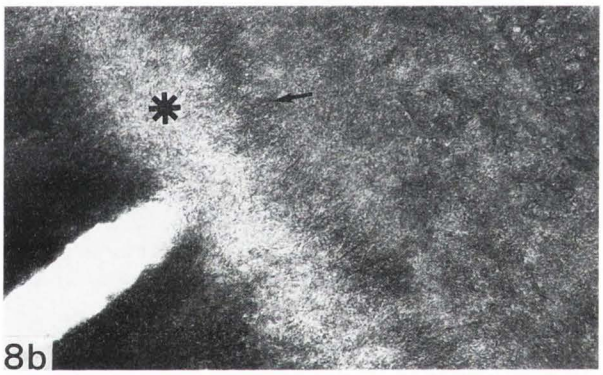
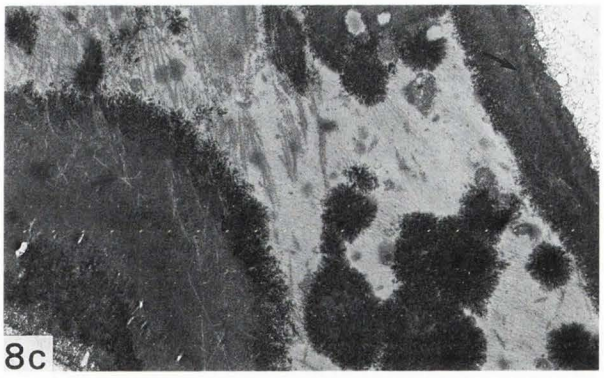


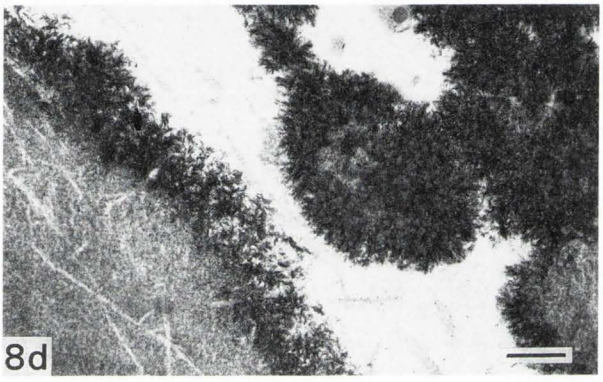
Figure 8 (at right). Transmission electron micrographs of 100% coated PLA after 8 weeks of culture showing direct deposition of mineralized extracellular matrix (ECM) onto the hydroxyapatite (HA); there is a gradual transition between the hydroxyapatite and the mineralized extracellular matrix. Note single electron dense layer (arrow) and electron lucent zone (asterisk) at the interface in (**a**). (**b**) is a higher magnification of (a). The electron dense layer is also present at the periphery of mineralization globules (**c, d**). Also note multiple electron dense layers (arrow) at the interface in (c). Bar = 0.6 μm (a), 0.2 μm (b), 1.4 μm (c), 0.6 μm (d).

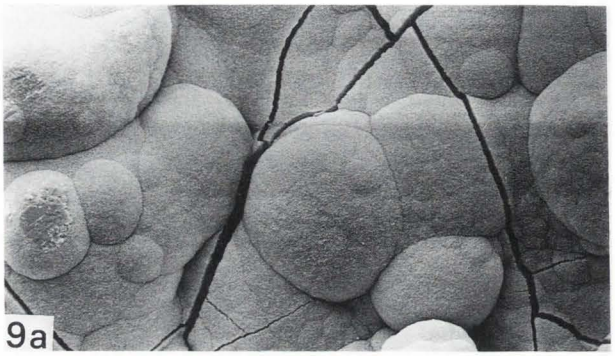
accretions to which collagen fibres were attached (Fig. 6). In addition to the formation of a mineralized extracellular matrix on the hydroxyapatite particles, calcium and phosphorous containing (as revealed by X-ray microanalysis, but not shown herein) mineralization globules were also seen on the uncoated PLA surface of 15% and 36% coated PLA (Fig. 7). The formation of these globules, however, was more widespread with 36% coated, than with 15% coated PLA. Figure 8 shows transmission electron micrographs of mineralized extracellular matrix on 100% coated PLA after an 8 week culture. At the interface, both electron lucent and electron dense layers can be seen (Figs. 8a, b). Also, single or alternating electron dense layers have been observed (Fig. 8c). Collagen fibres were frequently seen in the mineralized extracellular matrix.

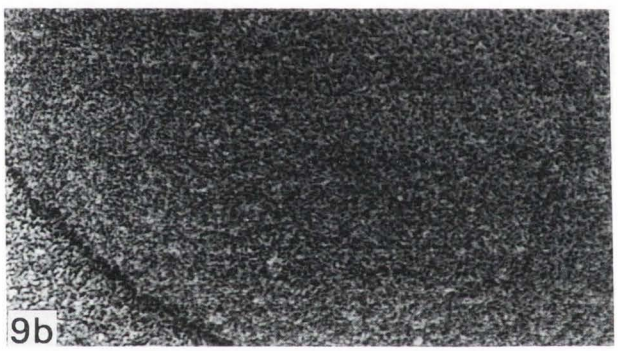


Cell free environment

When stained with toluidine blue or alcian blue, a distinct basophilic line was seen on the 100% coated PLA which was similar to that seen in the cell cultures. SEM examination revealed that the hydroxyapatite surface was composed of small plate-like crystals (Figs. 9a, b). When examined with TEM, a gradual transition was seen between the bulk hydroxyapatite material and the outer surface, which was composed of calcium and phosphorous containing, needle-shaped crystals (Fig. 9c). However, neither fibrous or globular mineralized material was attached to this layer.







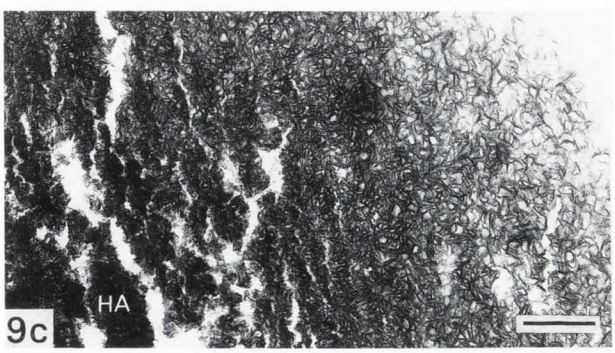


Figure 9. **(a, b)** Scanning electron micrograph of 100% coated PLA after 1 week in culture medium, in the absence of cells. Note the deposition of small plate-like crystals on the coating. **(c)** Transmission electron micrograph of the same coating, a gradual transition is seen from hydroxyapatite to needle-shaped crystals at the periphery. Bar = 11.1 μm (a), 1.5 μm (b), 0.4 μm (c).

Discussion

The results of this study indicate that a correlation exists between the percentage of hydroxyapatite coating on PLA and the time in which mineralized extracellular matrix is formed. This study has further shown that the presence of hydroxyapatite is required for the formation of a mineralized extracellular matrix on PLA. Although the interface formed was similar for all hydroxyapatite coatings, the time in which the extracellular matrix was formed, and its subsequent mineralization occurred, varied. A reason for this might be an alteration in cell phenotype expression induced by the amount of available hydroxyapatite in the composite (Khare *et al.*, 1990). In addition, as the different coating percentages were achieved by plasma spraying, the resulting differences in surface topography may also have influenced cellular activity (Cherhoudi *et al.*, 1989; Brunette *et al.*, 1991; Curtis and Clark, 1990). The review by Curtis and Clark (1990) indeed showed that surface topography will influence cells in a wide variety of ways, which include cell attachment and cell migration. Hence, the difference in cell migration and spreading over the uncoated or 100% coated PLA and 15% or 36% coated PLA may be controlled by the surface topography of the latter two materials. The influence of ions released from the hydroxyapatite and PLA substrata cannot be the reason for the differences in cell behaviour, as far as cell migration is concerned, as cells formed a confluent layer on both uncoated and 100% coated PLA.

Interpretation of the formed mineralized layers on 15%, 36% and 100% coated PLA in cell culture is difficult, as it was also observed to a certain extent on 100% coated PLA in a cell free environment. The presence of the calcified layer on the control specimens is in contrast to earlier studies (de Bruijn *et al.*, 1991) in which no alterations of the hydroxyapatite surface were seen when samples were placed in culture medium in the absence of cells. A reason for this discrepancy may be the amorphous character of the hydroxyapatite used in this study (Fig. 1d), as opposed to more crystalline hydroxyapatite used in previous studies. Evidence for this has been given by LeGeros (1988), who showed that crystallinity or crystal size influences the degradation properties of a material. We have also observed this in a recent study (de Bruijn *et al.*, 1992b, c); the more crystalline a material, the slower its ion release. Similar phenomena may also explain the more widespread presence of mineralization globules with 36% coated PLA than with 15% coated PLA. With the former material, a higher calcium and phosphorous ion concentration will be present in the culture medium due to the higher percentage of hydroxyapatite coating. In addition, the presence of PLA, which causes an acidic environment when degrading, will enhance hydroxyapatite dissolution (Lee *et al.*, 1989). The above described dissolution process of hydroxyapatite will, as hypothesized by Daculsi *et al.* (1990), result in a high calcium and phosphate ion concentration at the ceramic surface, followed by possible reprecipitation. Together with this reprecipitation process, serum proteins may be incorporated, which may account for the distinct basophilic line on the coating particles after the light microscopical toluidine blue or alcian blue staining. Thus, part of the observed mineralization may be due to degradation of the hydroxyapatite ceramic followed by reprecipitation, without cellular activity. However, we believe that apart from this physico-chemical dissolution-(re)precipitation process, a cell mediated mineralized extracellular matrix is also formed. Evidence is

shown in Figure 6, in which collagen fibre containing globular accretions are deposited on the hydroxyapatite particles. With only physiological mineralization, one would expect a layer that is more homogeneous in thickness. Another example, which may account for cell mediated mineralized extracellular matrix formation, is seen when one compares Figure 9b and Figure 4 of 100% coated PLA in a cell free and cell containing environment respectively; it is clear that the hydroxyapatite particles of both materials are covered with needle shaped crystals. However, in the cell containing environment, a mineralized globular matrix is formed on this crystal layer that shows similarities with early bone formation *in vitro* (Davies *et al.*, 1991). TEM examination of this interface revealed an electron dense layer interposed with the hydroxyapatite coating and the mineralized extracellular matrix. This electron dense layer was only present at the interface in the cell containing culture, and not in the cell free culture (compare Figure 9c and Figures 8a, b). This is similar to our earlier observations (de Bruijn *et al.*, 1991, 1992a, b) that showed an electron dense layer at the interface between hydroxyapatite and the surrounding mineralized extracellular matrix. This layer showed morphological similarities with the electron dense layer seen *in vivo* (Jarcho *et al.*, 1977; Denissen *et al.*, 1980; Tracy and Doremus, 1984; van Blitterswijk *et al.*, 1985; Ganeles *et al.*, 1985), between hydroxyapatite and bone tissue.

In conclusion, we can state that a physico-chemical process of coating dissolution-(re)precipitation, as well as cellular activity, was responsible for the observed mineralization. Furthermore, the hydroxyapatite coating itself creates differences in surface topography, which may alter cellular behaviour and the mineralization process. Therefore, using this model, it was not possible to determine the percentage of hydroxyapatite required in the composite to initiate bone bonding. However, the covering rate of hydroxyapatite on PLA influences mineralized extracellular matrix formation *in vitro* and is expected to lead to differences in tissue response *in vivo*.

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Discussion with Reviewers

R.Z. LeGeros: How was the amount of hydroxyapatite covering the PLA substrate controlled?
Authors: The samples were coated with hydroxyapatite by moving the plasma spray gun in a defined cycle, parallel to the PLA surface. Increasing covering rates were produced by a repeated sequence of cycles. For example, only two cycles gave rise to the 15% hydroxyapatite covering, whereas five cycles resulted in a 36% coverage. All test samples were successively plasma sprayed on the same day, with identical settings of the plasma spray robot. The only difference was the number of runs for each covering rate, thus minimizing diversity.

R.Z. LeGeros: Is there any significance to the observed difference in average diameter of the plasma sprayed hydroxyapatite particles shown in Figs. 1a, 1b, 1c?
Authors: No. As mentioned above, all covering rates were produced under identical conditions, and therefore, we do regard these differences to be of major significance. Consequently, however, the total available hydroxyapatite surface area with the increasing covering percentage will be a more critical parameter.

P. Ducheyne and A. El-Ghannam: It is well known that coating with the plasma spray method affects the hydroxyapatite structure. Did the authors characterize the hydroxyapatite before and after coating? A precise method of characterizing the calcium phosphate coating is described in our work [Ducheyne P, Radin S,

Heughebaert M, Heughebaert JC (1990) Calcium phosphate ceramic coatings on porous titanium: Effects of structure and composition on electrophoretic deposition, vacuum sintering, and *in vivo* dissolution, Biomaterials **11**: 244-254]. Determination of the various phases, their crystallinity and their relative content is needed.
Authors: The feed-stock hydroxyapatite powder used for plasma spraying was sintered, highly crystalline pure hydroxyapatite. Characterization of the applied coating was performed with X-ray diffraction (Fig. 1d) according to standard procedures of our group [Flach JS, Shimp LA, van Blitterswijk CA, de Groot K (1993) A calibrated method for crystallinity determination of hydroxyapatite coatings. In: Characterization and Performance of Calcium Phosphate Coatings for Implants. Horowitz E, Parr JE (eds.), ASTM STP 1196, Am. Soc. Testing and Materials, Philadelphia, in press] revealing an amorphous hydroxyapatite coating. The fact that there are no standardized characterization methods (as the results are also dependent on the apparatus used), we believe that our coating characterization is adequate.

R.Z. LeGeros: Is the X-ray diffraction pattern shown in Fig. 1d representative of the coatings on the three types of covering (15, 36, 100%)?
Authors: X-ray diffraction was only performed on hydroxyapatite obtained from the 100% covering rate, due to the high quantity of coating available. However, as all coverings were produced under identical conditions (see above), we regard the pattern shown in Fig. 1d to be representative for all coverings.

R.Z. LeGeros: Our analysis of coatings obtained by plasma-spraying hydroxyapatite ceramic on metal substrates showed variability of coating composition, especially in the amorphous calcium phosphate (ACP) / hydroxyapatite (HA) ratio. Your coating appears to have a substantial amount of ACP. Can you estimate the ACP/HA ratio in the three types of coating?
Authors: Although we have not measured the ACP/HA ratio, the coatings examined are indeed mainly composed of a highly amorphous hydroxyapatite phase (> 90%), which is indicated by the low peaks and peak broadening in Fig. 1d. This low crystallinity is predominantly the result of the relatively large distance between the plasma-spray gun and the PLA substrate. This is to avoid disintegration of the latter, which would occur as a result of heat from the plasma-spray gun.

U. Gross: How did you determine that the fibrillar substances, e.g., in Figs. 5 and 6, are collagen?
Authors: With both SEM and TEM, the characteristic banding pattern of collagen was frequently visible. Thus, although it was only characterized morphologically, and not with specific biochemical/immunological techniques, we consider this fibrillar material to be mainly composed of collagen fibres.

U. Gross: Why do you claim that the precipitation of the crystalline material, e.g., in Fig. 8c and 8d, is mediated by cells in your *in vitro* assay?
Authors: The crystalline material present onto the hydroxyapatite particles in cell culture showed a different morphology to the precipitated crystals in the cell free environment (compare Figs. 4 and 9). It is, however, conceivable that this layer is produced by a combination of cell mediated processes and dissolution/(re)precipitation, as discussed in the second paragraph of the discussion.

U. Gross: Do the precipitated needle-like crystals, e.g., in Fig. 8d and 9c correspond to calcium phosphates?
Authors: Yes. We have performed X-ray microanalysis on those precipitates which indeed showed that they were composed of calcium and phosphorous.

P. Ducheyne: The authors report no difference among their samples which have different hydroxyapatite coating ratios. In fact, the presence of glucocorticoid (dexamethasone) in the tissue culture media has a stimulating effect on the bone cells causing them to produce bone-like nodules (Maniatopoulos *et al.*, 1988). Thus it would be better to show the effect of various hydroxyapatite coating ratios to run the experiment without adding dexamethasone.
Authors: We do not agree. Differences are reported, in that the time in which a mineralized extracellular matrix is formed, varies. No bone-like tissue was formed on uncoated PLA, whereas it was delayed on the 15% hydroxyapatite covering, as opposed to the 36% and 100% hydroxyapatite covering. Bone marrow cells cultured in the absence of dexamethasone will not produce bone-like tissue (Maniatopoulos *et al.*, 1988). Therefore, we believe that the culture medium chosen is appropriate.

R.Z. LeGeros: I agree with your conclusion that combined physico-chemical processes (dissolution/precipitation) and cellular activity. For this reason, the coating composition, e.g., ACP/HA ratio, presence of highly soluble phases such as α- and ß-TCP (tricalcium phosphate), TTCP (tetracalcium phosphate) or CaO may affect cellular activity. Do you agree?
Authors: Yes. In a recently published study [de Bruijn *et al.* The bone-calcium phosphate interface *in vitro*. In: Advances in Biomaterials, 10, Biomaterial-Tissue Interfaces. Doherty PJ *et al.* (eds), 1992, Elsevier, p. 305-309; and de Bruijn *et al.*, 1992b], we observed different bone-biomaterial interfaces with various types of calcium phosphates. For example, very little mineralized tissue was deposited on the surface of fluorapatite, whereas tetracalcium phosphate, (partially amorphous) hydroxyapatite and tricalcium phosphate showed extensive calcification.